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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Isabel Maria Cunha Lopes, Investigadora Principal do Centro de Estudos do Ambiente e do Mar e Departamento de Biologia da Universidade de Aveiro, e co-orientação da Doutora Graça Lopes, Professora Auxiliar do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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palavras-chave Herbicida, Fungicida, Reprodução, Espermatozóides de bovino, Toxicidade, Alternativa à experimentação animal

resumo A utilização de produtos fitossanitários aumentou exponencialmente nas ultimas décadas. O sulfato de cobre e o glifosato são dois pesticidas habitualmente utilizados, o primeiro como fungicida e o segundo como herbicida. Em explorações agropecuárias, os animais podem ser expostos a este tipo de produtos de várias maneiras: i) durante a sua aplicação algumas partículas podem espalhar-se e ser inaladas ou absorvidas pela derme ou ii) pela ingestão de alimentos e/ou água contaminados. Esta exposição pode causar efeitos adversos no ciclo reprodutivo destes animais. Na realidade, os espermatozóides são extremamente sensíveis a pequenas variações no organismo. A interação entre o produto químico e o esperma pode alterar sua mobilidade; velocidade e / ou viabilidade dependendo de que estruturas celulares são afetadas. O objetivo deste trabalho foi avaliar a toxicidade de concentrações de sulfato de cobre e glifosato em espermatozóides de bovino. Sémen comercial congelado de cinco touros diferentes foi exposto a três concentrações diferentes dos dois pesticidas, diluídos em tampão fosfato-salino (PBS), mais um controlo (PBS). Por cada touro, foram feitas três réplicas. Os parâmetros de motilidade e velocidade foram avaliados com recurso a um programa informático para análise de sémen e a viabilidade foi avaliada em esfergaços de sémen corados com eosina-nigrosina. Estes parâmetros foram determinados após 0, 30 e 90 minutos de exposição. O cobre provocou uma redução significativa na velocidades dos esperamatozóides na concentração mais alta que foi testada e após 90 minutos de exposição. Mais ainda, observou-se que na concentração de cobre mais baixa (e no tempo zero) os espermatozóides apresentaram maior mobilidade e velocidade do que as outras concentrações, sugerindo que o cobre pode aumentar a mobilidade dos espermatozóides em baixas concentrações. O glifosato reduziu significativamente a mobilidade e a viabilidade dos espermatozóides. Os resultados *in vitro* apresentam algumas limitações mas constituem ferramentas relevantes nas primeiras fases de avaliação de risco de compostos químicos, evitando a realização de experimentação animal.

keywords Herbicide, Fungicide, Reproduction, Bovine spermatozoa, Toxicity, Animal testing alternative

Abstract

The use of plant protection products has exponentially increased in the agricultural sector over the past decades. Copper sulfate and glyphosate are two commonly used pesticides, the former as a fungicide and the latter as an herbicide. Farm animals may be exposed to this type of products through different ways: i) the drift of pesticides during their application may lead to inhalation or dermic exposure or ii) through the ingestion of contaminated food or water. This exposure may lead to adverse effects in the reproduction of those animals. Actually, spermatozoa are extremely sensitive to slight variations in the organism. The interaction between the chemical and the sperm may alter its motility; velocity and/or viability depending on which cell structures are affected. The goal of this work was to assess the toxicity of copper sulfate and glyphosate concentrations on bovine spermatozoa. Commercial frozen semen from five different bulls were exposed to three different concentrations of the two pesticides, diluted in phosphate-buffered saline (PBS), plus a control (PBS). For each bull, three replicates were made. Motility and velocity endpoints were evaluated using a computer-assisted sperm analyzer and viability was determined in eosin-nigrosin staining smears. Endpoints were measured at 0, 30 and 90 minutes. Copper induced significant changes in the velocity of spermatozoa exposed to a concentration of 62.5 mg/L for 90 minutes. Adding to this, spermatozoa exposed to the lowest copper concentration (at time zero) showed higher motility and velocity than the other treatments, suggesting that copper may enhance motility at low concentrations. Glyphosate significantly reduced the motility and viability of spermatozoa. *In vitro* results are limited, but they are a good starting point for unveiling primary mechanisms of toxicity without the need to use living beings.

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Introduction

1.1 Environmental contamination

The world population has been increasing tremendously over the last century. This population growth led to an increase in industry, food production and earth resources consumption (e.g. Water, land, fossil fuels, etc.). In result, there was also an increase in waste produced, water pollutants and greenhouse gases (Carvalho, 2017). The present year the world population reached 7.5 billion people. By 2050, the United Nations estimates that this number will rise to 9.2 billion (United Nations, 2017). In parallel to such population increase a rapid industrialization and urbanization is occurring.

Over the years, there's been an increasing concern about environmental contamination, species extinction, and global public health. Anthropogenic activities such as industry, agriculture, and urban activities, are among the main source of environmental contamination (Callender and Rice, 2000), habitat loss and climate change (Chen, 2007). Industrial emissions from power plants or metallurgical industry, road dust from traffic emissions are some examples of anthropogenic pollution and environment degradation in urban areas (Wei and Yang, 2010).

A large number of pollutants from anthropogenic activities find their way into environmental compartments like soil, water, and plants. They enter the environment and impair the welfare of living organisms, reducing their fitness and, depending on their concentration, leading them to death (Duruibe et al., 2007). Some products are more noxious than others posing different intensity of risk for the environment and human health (Bi et al., 2006).

Another downside of human population growth is the need to increase food production. Earth resources are limited, and the land needed to produce food has increased with world population growth. The need to produce more from the same resources increased the use of agrochemicals which became an important component in agricultural fields, helping with crops protection from pests and more food production (Hester and Harrison, 2016). According to the European Commission (2016b):

"A 'pesticide' is something that prevents, destroys, or controls a harmful organism ('pest')

or disease, or protects plants or plant products during production, storage, and transport."

The term pesticide includes herbicides, fungicides, insecticides, growth regulators, amongst others. The ingredients that form a pesticide are divided into two categories: i) "active ingredients", the chemicals in a pesticide responsible for pest control, they can repel or destroy pests, or act as plant regulators; ii) "inert ingredients", substances present in the formulations that enhance product performance, for example, they can help with surface penetration, extension of shelf-life or improve safety for the user (European Commission, 2016b).

The use of pesticides has contributed to an increase in long-term productivity of agricultural land and food production. However, pesticides do not always decrease crop losses. For instance, the continuous plantation of corn led to an increase in insect plagues, and consequently corn losses, even though the application rate of insecticides had increased too (Pimentel, 2005). The excess use of pesticides can lead to pest resistance and at the same time can unintentionally destroy beneficial predators of this pests (Wilson and Tisdell, 2001).

Reports of chemical contamination of soil and water by pesticides and fertilizers, the death of non-target organisms and side effects in human population have been growing (Aktar et al., 2009). Often, rain events promote the relocation of pesticides residues from the application site to other terrestrial and aquatic systems as shown in figure 1. They spread around the application site by runoffs, increasing the chance to toxically harm non-target organisms (Chiovarou and Siewicki, 2008).

In agricultural fields, pesticides can drift during application, leach from contaminated soils during rain events or simply be washed off the plants during irrigation periods, and subsequently be transported through runoff channels to near water bodies or uncontaminated soils (Cessna et al., 2005). In addition, pesticides are not always handled correctly. If packaging disposal, filling of sprayers and cleaning the application material and transportation tanks are not done properly, pesticide residues may end up being washed off to the sewers or into surface waters (Gerecke et al., 2002). Some pesticides are used in

non-agricultural sites, like sidewalks, roads, railways or gardens, to reduce weed growth, and its residues are often transported to the sewers by rain events (Reichenberger et al., 2007). Run-offs, wastewater discharges, and spills are for that reason the main sources of surface and groundwater contamination by pesticides (Cessna et al., 2005).

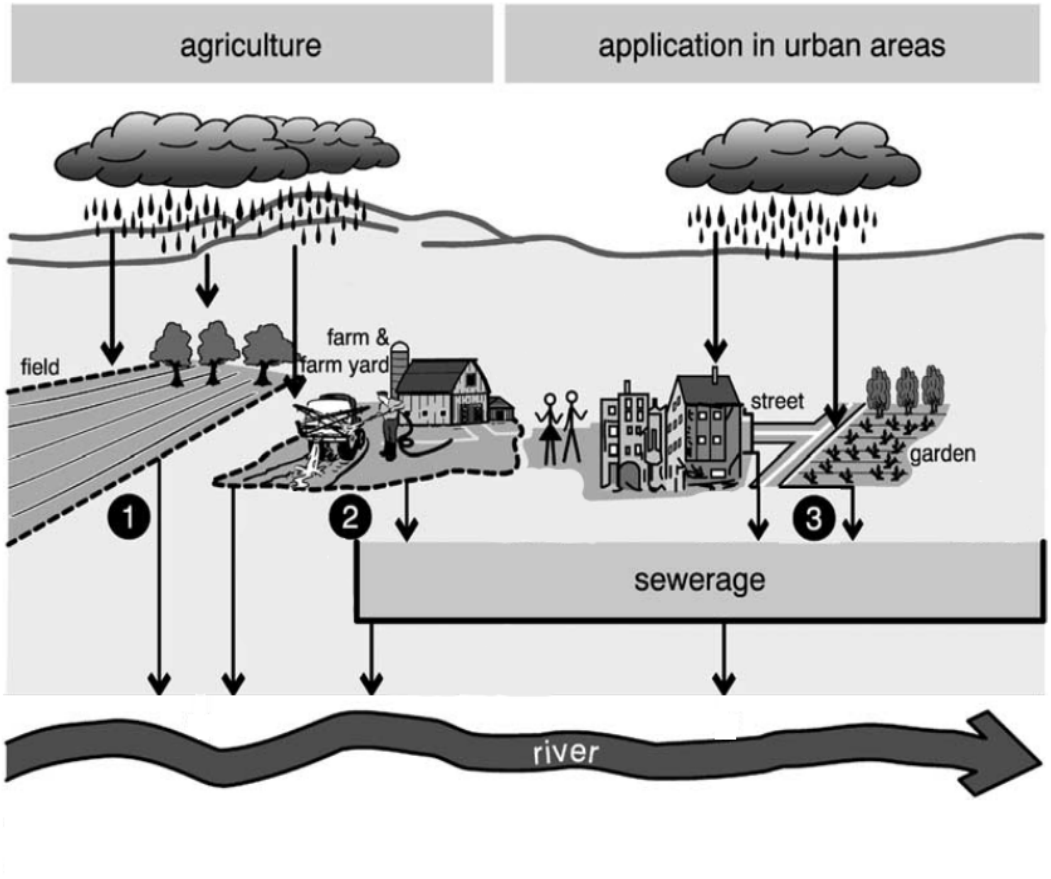

Figure 1: Common pesticide application sites and it's translocation to near water bodies. 1) agricultural fields; 2) inappropriate disposal of pesticide residues before/after application; 3) pesticide use in urban areas (e.g. lawns, streets, sidewalks, etc.) (Image adapted from Gerecke et al. 2002).

When pesticides enter water bodies, they may bioaccumulate in living organisms, passing through the whole food chain (Okoro et al., 2011). At high concentrations they may induce acute toxicity, causing lethal effects to organisms from different trophic levels (from producers to secondary consumers) (Mineau and Whiteside, 2013). Important studies have

been made to understand the behavioral of these chemicals in the environment and their long-term effects on non-target organisms. These studies have helped regulate the use of pesticides around the world (Scholz et al., 2013). The European Commission has established values of Maximum Residue Levels (MRL: the highest level of a pesticide residue that is legally tolerated in or on food or feed when pesticides are applied correctly (Good Agricultural Practice)) to be found in the food of human and animals (European Commission, 2017).

Food originated from plants is commonly grown using pesticides, which poses a risk to human health. High levels of some pesticides in this type of food items have been associated with a higher incidence of several human diseases, such as cancer, Alzheimer's and Parkinson (Khaniki, 2007). Food originated from animal sources can also be contaminated by pesticides (Nag and Raikwa, 2011, Nag and Raikwar, 2008). Animals can be exposed to pesticides by aerial drift during application, water contamination or presence of residues in their food (Tsiplakou et al., 2010). However, good agricultural practices and selection of less persistent substances can reduce pesticide residues in animal food to levels below MRL (Kan, 2004). When ingested, depending on their physicochemical properties, toxic substances like pesticides can be metabolized into other substances or not metabolized at all. For example, metals are not metabolized by the organism, if they are not excreted they may bind to body tissues like the liver, kidneys or bones (Kan and Meijer, 2007). Many studies have found pesticides residues in bovine milk, which is a public health concern, since adults and children consume milk all around the world (Darko and Acquaah, 2008, Khaniki, 2007, Pagliuca et al., 2006, Sharma et al., 2007).

1.2 Test chemicals

1.2.1 Copper Sulfate

Copper (Cu) is a naturally occurring element. It is relatively abundant on Earth and is one of the most used metals by men (e.g. agriculture, electrical wires, plumbing), for

that reason, it is widely extracted from mines (Astdr, 2004). In the environment, Cu is moderately soluble in water, has the ability to bioaccumulate and binds easily to sediments and organic matter, potentiating its toxic effects (Wang, 1987). Its bioavailability depends on many environmental factors (e.g. pH, redox potential, sediment type) (Fleming and Trevors, 1989).

Copper is also an essential trace element required in small amounts by most organisms. It functions as a cofactor and is required by a variety of important enzymes in order for them to function properly (Turnlund, 1988). These enzymes have an important role in growth, development, and maintenance of the organism (Turnlund, 1998). Low levels of copper can have adverse effects like anemia, myocardial disease, and osteoporosis (Williams, 1983). On the other hand, high concentrations of this metal are toxic and can be fatal. Copper overload causes oxidative damage to lipids, proteins, and DNA which interferes with important cell functions (Gaetke and Chow, 2003, McCluggage, 1991, Nolan, 1983).

Chronic exposure to copper affects the liver, the kidney, the brain and other organs (Gaetke and Chow, 2003). Liver and kidney toxicity is associated with irreversible nuclear damage leading to cell death (Stern, 2010). Cu has many uses, it can be present in everyday items like pipes, machinery, pesticides and intrauterine devices. Contact with these items may not be harmful but prolonged exposure is damaging to the organism (Barceloux, 1999). Cu is known to be an effective biocide, but like most pesticides, it may also affect non-target organisms. Cu ions have the ability to bind to groups of proteins in fungi and algae and cause cell damage and leakage (U.S. Environmental Protection Agency, 2009b). Copper has its role in oxidative stress, it can catalyze free radicals but, it can also help reduce reactive oxygen species (Theophanides and Anastassopoulou, 2002).

Copper sulfate pentahydrate ($CuSO_4 \cdot 5\,H_2O$) is an inorganic salt, in the form of blue crystals or powder, soluble in water (National Center for Biotechnology Information, 2016). These salts are more toxic than insoluble compounds (World Health Orgnization, 2004). Copper salts are introduced into water bodies to control algae growth and molluscicides and used in agriculture as copper-based fungicides (World Health Orgnization, 1993). When it enters freshwater systems, it affects plankton which may lead to dramatic changes in

the food web (Oliveira-Filho et al., 2004). Agriculture run-offs and urban application sites may be sources of water and soil contamination by this metal (Okoro et al., 2011).

Copper sulfate is also used in dairy animals, for hoof baths, to treat and prevent digital dermatitis (Thomsen, 2015). The presence of pathogens and algae in fish production can also be treated with soluble copper (Wang et al., 2015), even though, it can change behavior (Heath, 1995), immunological function, swimming performance (Langston and Bebianno, 1998) and enzyme activities in fish.

Exposure to metals is long associated with negative effects on the reproductive system. High concentrations of copper lead to low spermatozoa motility and density, and increased morphological anomalies in human sperm exposed to a range of concentrations between 10 ng/ml and 100 mg/ml (Roblero et al., 1996). Motility of spermatozoa depends on the mitochondrial activity, which can be impaired by the excess of copper (Kňažická et al., 2012a).

Due to its application in water reservoirs, the World Health Orgnization (2004) set a guideline value for the presence of copper in drinking water in 2 mg/L. In 2009, this value was reduced to 1.3 mg/L by U.S. Environmental Protection Agency (2009a), which is considered protective against adverse copper effects.

1.2.2 Glyphosate

Between 2008 and 2012 the most sold pesticides worldwide were herbicides, most of them for agricultural purposes. In 2012, the most commonly used pesticide in the U.S. agricultural market was glyphosate, approximately 270-290 million pounds of active ingredient compared to the next on the list, Atrazine, with 64-74 million pounds of active ingredient (US EPA, 2017).

Glyphosate is the main ingredient in Roundup® herbicide produced by Monsanto, and it was first introduced in 1974 for weed control (Franz et al., 1997). Roundup® formulations usually contain glyphosate in the form of an isopropylamine salt and various adjuvants, also known as inert ingredients, that help the product penetrate the plant (Webster et al.,

2014).

Glyphosate-based formulations are the most used herbicides in the world. They are broad-spectrum, non-selective herbicides that interfere with the shikimic acid pathway through inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) (Cerdeira and Duke, 2006). The deregulation of this pathway leads to the accumulation of high levels of shikimic acid and its derivatives and the reduction of aromatic amino acids synthesized, inhibiting plant growth (Piola et al., 2013). According to Williams et al. (2000), the shikimic pathway exists only in plants reason why this herbicide shouldn't be harmful to other non-target organisms. Glyphosate was considered environmentally safe due to its rapid biodegradation, strong adsorption to soil and microbial degradation (Webster et al., 2014).

With the beneficial effects of glyphosate in weed control, its use has increased exponentially over the years. New transgenic herbicide-resistant crops have been engineered to support large amounts of this herbicide, this crops are more productive, which brings benefits for the farmers (Rani and Usha, 2013). Since these plants do not metabolize glyphosate, they accumulate it in the leaves, grains, and fruits (Arregui et al., 2004). Reports of weed resistance to glyphosate have been growing up. To avoid the spread of these weeds, farmers increase the application rates and reduce the time between applications, loading the fields with excessive concentrations of glyphosate (United States Department of Agriculture, 2017).

A study done with animals fed with transgenic plants showed residues of glyphosate (between 15 ng/g and 30ng/g) in the urine, kidney, liver, intestine, and muscles (Krüger et al., 2013). Krüger et al. (2014) also found residues of glyphosate in human urine (maximum value found 71.3 μg/ml). Even though the target mechanism of action of glyphosate is specific to plants, some adverse effects have been reported in non-target organisms. A study with earthworms and Roundup® showed that glyphosate can decrease survival and cocoon production (Stellin et al., 2017). Glyphosate can also cause reproductive toxicity to fish, reducing egg production and early-stage embryo mortality (Webster et al., 2014) and in amphibians (Mann and Bidwell, 1999, Relyea and Jones, 2009). Glyphosate

and Roundup® can decrease the activity of the cytochrome P450 aromatase in human cells (Richard et al., 2005), induce oxidative stress leading to cell death in Sertoli cells from rats (Cavalli et al., 2013) and adversely affect reproduction, reducing human sperm motility (Anifrandis et al., 2016). In 2015, after an extensive review of animal studies exposed to glyphosate, the International Agency for Research on Cancer (IARC) changed the glyphosate category to *"probably carcinogenic to humans"*. This means there is limited evidence of carcinogenicity in humans, but, at the same time, there is sufficient evidence of a positive association between the toxic agent and cancer in animals (International Agency for Research on Cancer, 2015)

There is an assumption that glyphosate itself is the most toxic compound to non-target organisms in all the formulations available in the market. Even though glyphosate is never applied alone (it is part of a formulation with adjuvants), for regulatory measures it is the only compound tested. Adjuvants are always considered an inert ingredient in glyphosate formulations, but studies have found that the mixture in the glyphosate formulations is a lot more toxic to non-target organisms, then the glyphosate alone (Mesnage et al., 2013, 2015, Richard et al., 2005).

1.3 Alternative methods to animal experimentation

The EU regulatory framework for the Registration, Evaluation and Authorization of Chemicals (REACH) was created in 2007 due to the lack of information on hazards to human health and to the environment of many of the chemicals released to the market over the years. This new legislation required new animal testing since non-animal methods weren't solely reliable (European Commission, 2016c).

The use of animals in scientific research is common and the ethical concern about animal welfare has been growing in the society. Over the last years, the development of new alternative methods to animal experimentation has been increasing. The "3R's" principles, defined by Russell and Burch (1959), state that alternative methods in toxicology testing must be able to:

- Reduce the number of animals in experimental procedures;
- Refine procedures, so they become less painful and less stressful for the animals;
- Replace laboratory animal testing with *in vitro*, *ex-vivo* or *in silico* tests.

The 3R's principles have been supported by laws in the EU since 1986 (Directive 86/609/EEC) with the purpose to protect animals used in scientific experimentation, and they required that available alternatives were tested before animal experimentation is conducted (Council of the European Communities, 1986). As the article 7.2 of this directive states:

"An experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available. "

In the light of this directive, some non-animal alternative methods for toxicity testing started to be validated. This validation consists in the demonstration of reliability and relevance of a method for a certain purpose (Bruner et al., 1998). So, in 1991, the European Centre for the Validation of Alternative Methods (ECVAM) was created to evaluate and validate alternative methods and to create a database with all the approved *in vitro* tests (Kandárová and Letašiová, 2011).

In 2010, the European Union revised the Directive 86/609/EEC, releasing a more extensive directive "on the protection of animals used for scientific purposes". This directive established some new rules about "the replacement and reduction of the use of animals in procedures and the refinement of the breeding, accommodation, care and use of animals in procedures" (European Union, 2010) and about the use of some invertebrate species and mammalian fetuses (European Commission, 2016a).

Over the years, significant efforts have been done to develop alternative methods that are reliable, sensitive and predictable, but only a few have been accepted and implemented (Adler et al., 2010). This happens due to the complexity of physiological mechanisms,

multiple organ and tissue interaction that can't be replaced by single cells testing. However, numerous *in vitro* systems have been developed for cell-specific toxicity, like hepatotoxicity and neurotoxicity. For reproduction toxicity, there are several models like whole-embryo culture assay, Sertoli cell lines, Leydig cell lines, FETAX (Frog Embryo Teratogenesis Assay Xenopus) and sperm motility (Adler et al., 2010, Lorenzetti et al., 2011, Worth and Balls, 2002).

In the reproductive toxicity assessment, the existing alternative models are unable to cover all the parts of the mechanism involved in the reproduction. However, they can provide detailed information on mechanisms of toxicity and they can be combined with existing *in vivo* tests to reduce the number of animals used (Scholz et al., 2013).

1.3.1 Computer Assisted Semen Analysis

One of the alternatives in male reproductive toxicity assessment is the use of spermatozoa. Spermatozoa have an important role in fertilization and for that, their cellular structure must be intact, they must be mobile and able to deliver the intact male genome to the oocyte. Sperm motility and cell integrity are for that reason, key parameters to evaluate male fertility (Larsen et al., 2000). The potential toxic effects of a chemical on sperm can then be assessed by *in vitro* exposure to the test substance and evaluation of the key parameters.

The use of the CASA (computer-assisted semen analysis) system makes it possible to evaluate quantitatively the sperm motility and velocity and assess the overall performance of sperm motion through videomicrography (EURL ECVAM DB-ALM, 2010). Viability must also be assessed by techniques that show the integrity of the cell, like a hypoosmotic swelling test, eosin-nigrosin, trypan blue and others. CASA is now considered the standard tool to assess sperm motility (Contri et al., 2010).

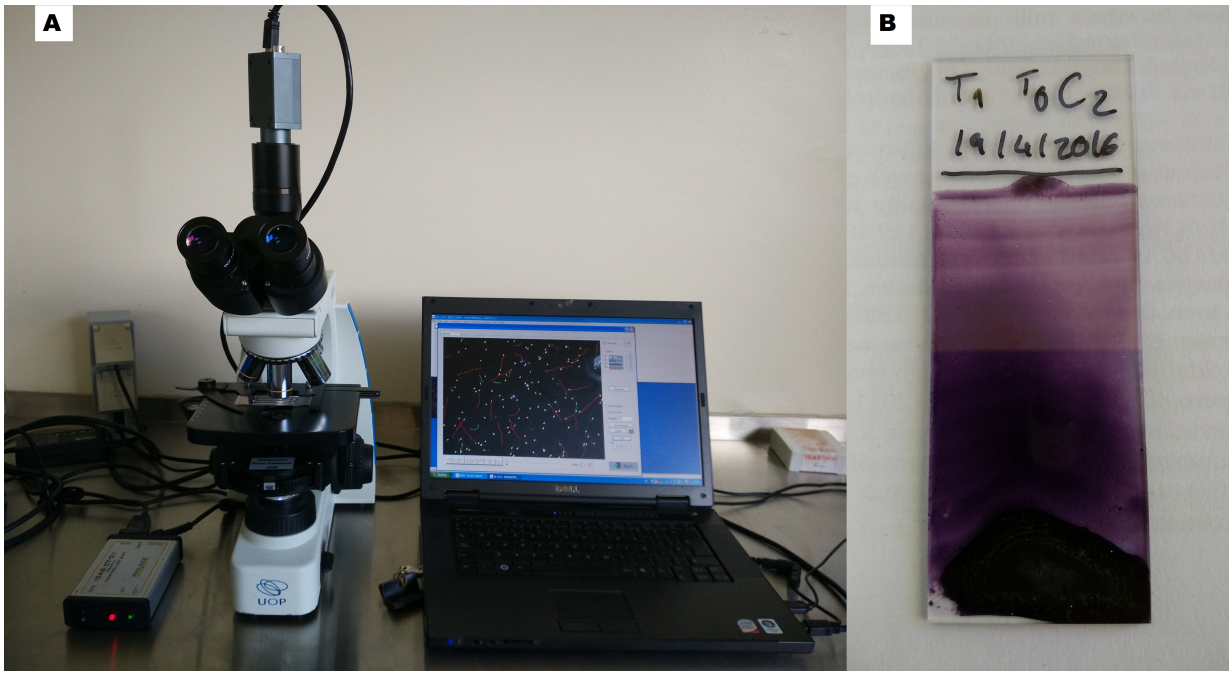

Figure 2: A) CASA setup for the motility analysis. It consists on a microscope, with a warm plate, connected to a computer through a camera. The software records the movement of the sperm heads and calculates all the motility parameters; B) Eosin-nigrosin staining for viability evaluation.

The CASA setup for the motility analysis, consists of a microscope, with a warm plate, connected to a computer through a camera as showed in figure 2 (A). The software records the movement of the sperm heads and calculates all the motility parameters. In the same figure (B) is an Eosin-nigrosin stain slide, used for viability evaluation. The nigrosin makes the sperm heads easier to visualize by increasing its contrast with the background. At the same time, the eosin makes it possible to distinguish between dead and live sperm by sating dead cells, when observed under a microscope dead sperm is stained dark pink while alive sperm appears white (Agarwal et al., 2016).

For laboratory experiments using CASA any spermatozoa can be used (bovine, rat, fish, human, stallion or others). Although human sperm is hard to obtain, since it depends on the will of volunteers, has low ejaculate volume and low sperm density (Seibert, 1992). For routinely cytotoxic testing bovine sperm is easier to obtain, almost painless to the

animal and the ejaculates have higher volume. Due to its high-volume ejaculates, bovine semen can be stored in liquid nitrogen for future use. Frozen bovine semen straws are also available commercially (Worth and Balls, 2002).

1.4 Objectives

The aim of this work was to study the effects of two widely used pesticides, copper sulfate, and glyphosate, on male reproductivity. These pesticides are known to cause reproductive and developmental toxicity and many of *in vivo* assays have been done to evaluate it (Cavalli et al., 2013, Dallegrave et al., 2003, Lopes et al., 2014, Perkins et al., 2000, Roychoudhury et al., 2015, Santos et al., 2013). As mentioned above, the need to reduce the number of animals in toxicity testing is a priority, and for that reason, this study was performed using a non-animal approach with mammalian spermatozoa. Two assays were performed, one for each pesticide, using a CASA system (based in the DB-ALM protocol nº21: Computer-assisted Semen Analysis (EURL ECVAM DB-ALM, 2010)) and a viability test with eosin-nigrosin.

Materials and methods

2.1 Test chemicals

Copper sulphate ($CuSO_4 \cdot 5\,H_2O$ and CAS number 7758-99-8) was purchased as a powder to Sigma-Aldrich (St. Louis, USA). Three stock solution of 5, 62.5 and 125 mg/L of Cu were prepared by diluting copper sulphate with a phosphate buffered solution (PBS). A volume of 0.25 ml of bovine sperm (in PBS as well) was then added to these stock concentrations, so that the following final nominal concentrations were tested: 2.5, 31.25 and 62.5 mg/L (Figure 3). These concentrations of copper were selected based on existing studies with bovine spermatozoa (Kňažická et al., 2012a,b) and human spermatozoa (Roblero et al., 1996)

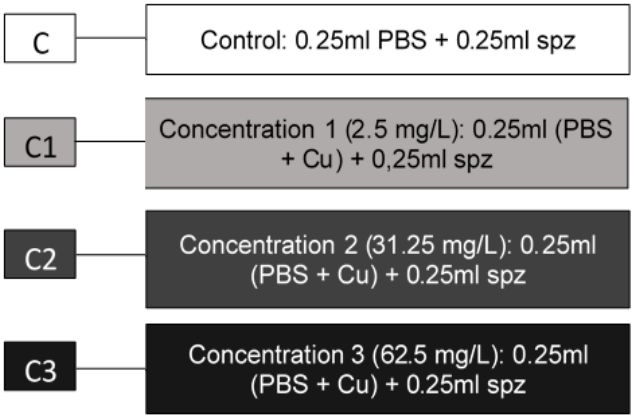

Figure 3: Volume and content of each concentration of copper.

Glyphosate was acquired as the commercial formulation of Roundup® (Monsanto). It contains 7.2g/l of glyphosate [N-(Phosphonomethyl) glycine; molecular formula $C_3H_8NO_5P$; CAS nº 1071-83-6] in the form of an isoproylamine salt. Roundup® was diluted in PBS to create three stock solutions of 1.44, 14.4 and 720 mg/L of the a.i. glyphosate. The final nominal concentrations after adding the sperm were 0.72, 7.2 and 360 mg/L of a.i. glyphosate (Figure 4). These concentrations were chosen based on previous studies with sertoli cells (Cavalli et al., 2013), hepatomacell line HepG2 (Gasnier et al., 2009) and human urine (Krüger et al., 2014).

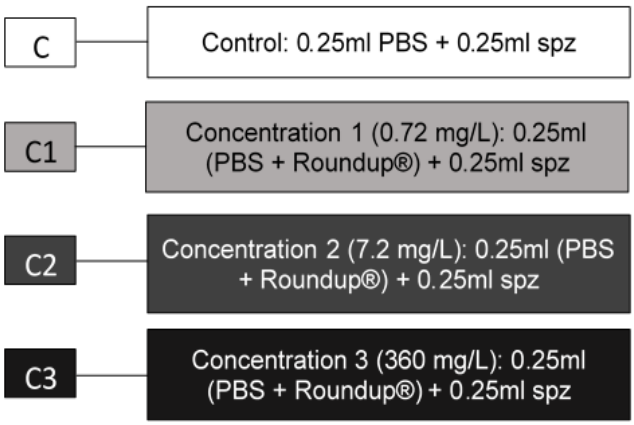

Figure 4: Volume and content of each concentration of Roundup®.

2.2 *In vitro* assays with spermatozoa

Frozen-thawed commercial semen from five different bulls, stored in 0.25 ml plastic straws, was used to perform the assays. For each bull, three replicates were made, and for each replicate, five straws were used. Every straw was thawed at 37ºC for 30 seconds and then mixed with the others. The semen was then centrifuged for 5 minutes at 720 g and resuspended in 1 ml of warm (37ºC) PBS. Before starting the experiment, a sample straw of each bull was evaluated; the number of spermatozoa in a straw was counted in a Neubauer chamber. Each bull straw had approximately $20x10^6$ spz/ml. Adding to this, subjective motility was observed under a microscope and was approximately 45% for every sample. The eosin-nigrosin staining procedure was used to evaluate the viability of the sperm.

For each bull sperm, four Eppendorf were prepared. One for the control, with 0.25 ml of PBS, and the other three with 0.25 ml of each of the three concentrations of copper or glyphosate to be tested. Each solution was then warmed to 37ºC to receive the spermatozoa suspensions. After being resuspended in PBS the spermatozoa were added to each Eppendorf, 0.25ml spermatozoa per treatment. During the assay, all the material used to handle the semen was at 37ºC.

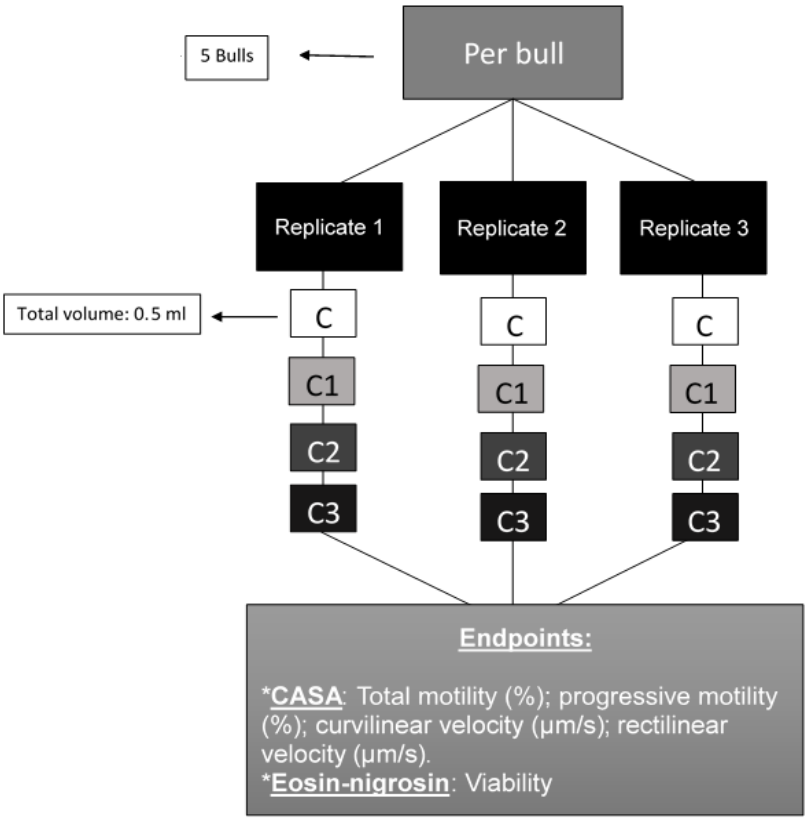

Figure 5: Experimental design layout with the number of replicates, bulls, and treatments; endpoints for each evaluation method; and volume of each concentration.

The following parameters were measured after 0 min, 30min, and 90 min of exposure: motility, velocity and viability of spermatozoa. These endpoints were measured by using two methodologies: CASA was used to assess total percentage of motile spermatozoa, percentage of progressive motile spermatozoa, rectilinear velocity (μm/s) and curvilinear velocity (μm/s) while eosin-nigrosin was used to evaluate the viability (live/dead sperm count). For the CASA, each 10 μl sample was placed on a slide and covered with a glass coverslip. Each slide was individually observed under a microscope (UB203i phase contrast microscope, magnification x100) with heated stage, and all the motility parameters were registered by the software (ISAS®; Proiser, Valencia, Spain).

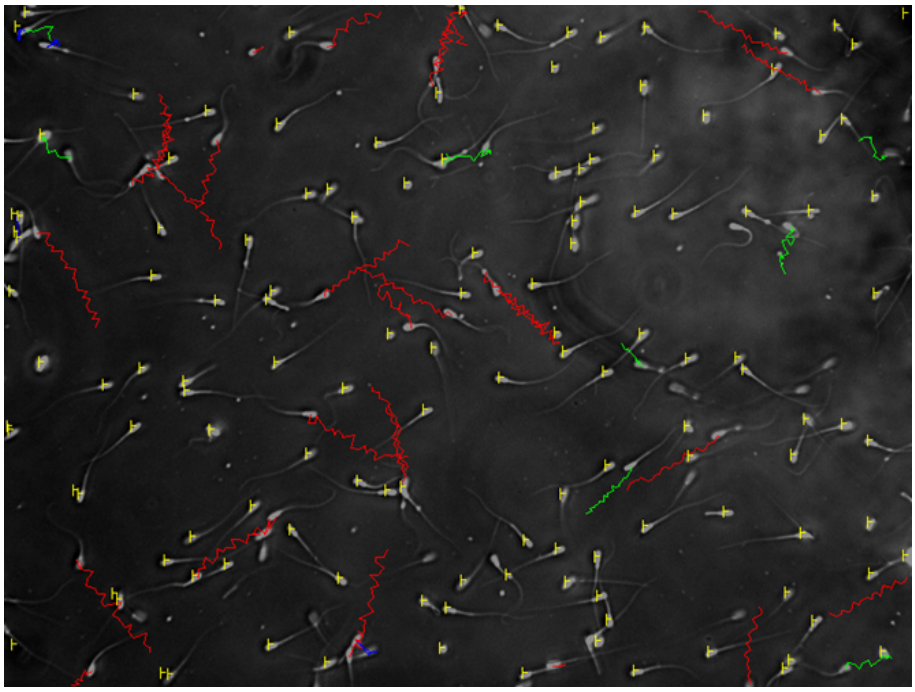

Figure 6: Spermatozoa head movement tracked with CASA software. Different colors symbolize different types of velocities. Red: fast; Green: medium; Blue: slow; Yellow: immobile.

For the eosin-nigrosin stains, each 10 μl sample was gently mixed with 10 μl of eosin-nigrosin, spread across a slide and allowed to dry on a heated plate. In the end of the experiment, all the slices were observed under a light microscope (UB203i, magnification x1000), and 100 live/dead spermatozoa were counted.

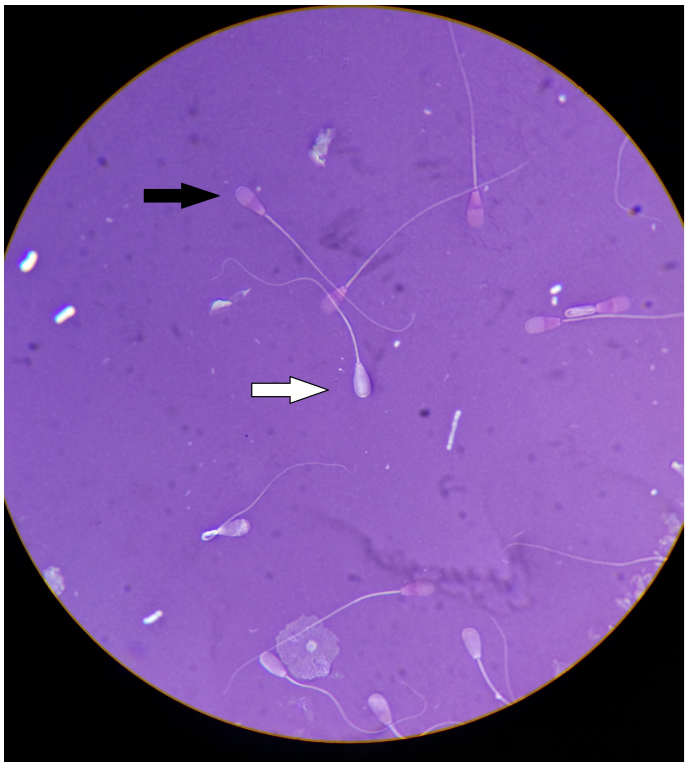

Figure 7: Spermatozoa stained with eosin-nigrosin observed under a light microscope (UB203i, magnification x1000). White arrow: live sperm; Black arrow: dead sperm.

2.3 Statistical analysis

A two-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparison test were performed to evaluate significant differences between concentrations of the pesticides and the respective control ($p<0.05$) at different evaluation times. The normality of data and homoscedasticity of variance were firstly tested with the Shapiro-Wilk's and Bartlett's tests, respectively.

The software SigmaPlot v.12.5 from the company Systat Software Inc. was used to perform data analysis of motility and viability.

Results

3.1 *In vitro* assay with copper sulfate

A slight decrease was observed in the total and progressive motility and viability of spermatozoa when exposed to increasing concentrations of copper, specially, at the highest exposure period (90 minutes), though this decrease was not significant comparatively to the control ($p>0.05$; Figures 8 and 10).

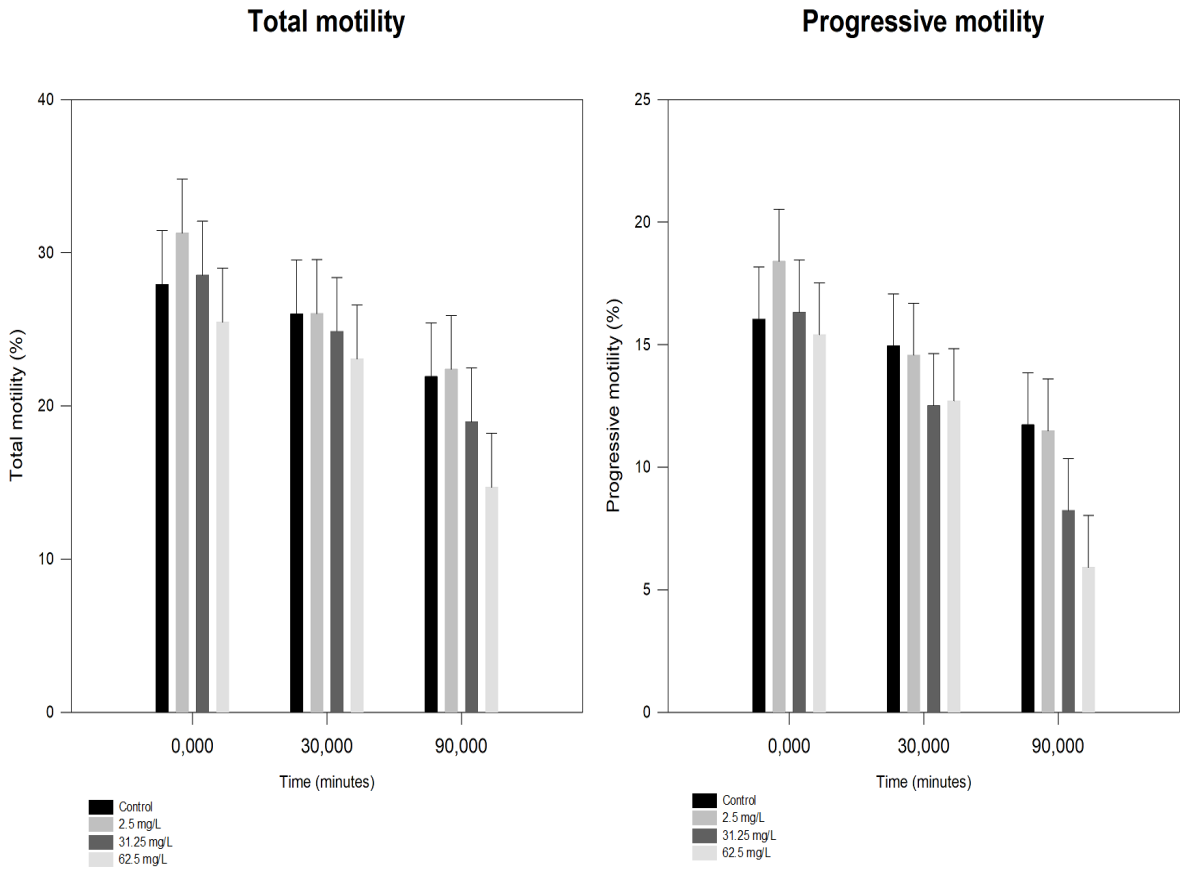

Figure 8: Percentage of total and progressive motility of bovine spermatozoa after being exposed to different Cu concentrations.

However, the highest tested concentration of copper significantly reduced (in more than 20 and 40%, respectively) the curvilinear and rectilinear velocity of spermatozoa relatively to the controls, after an exposure of 90 minutes ($p<0.001$; Figure 9).

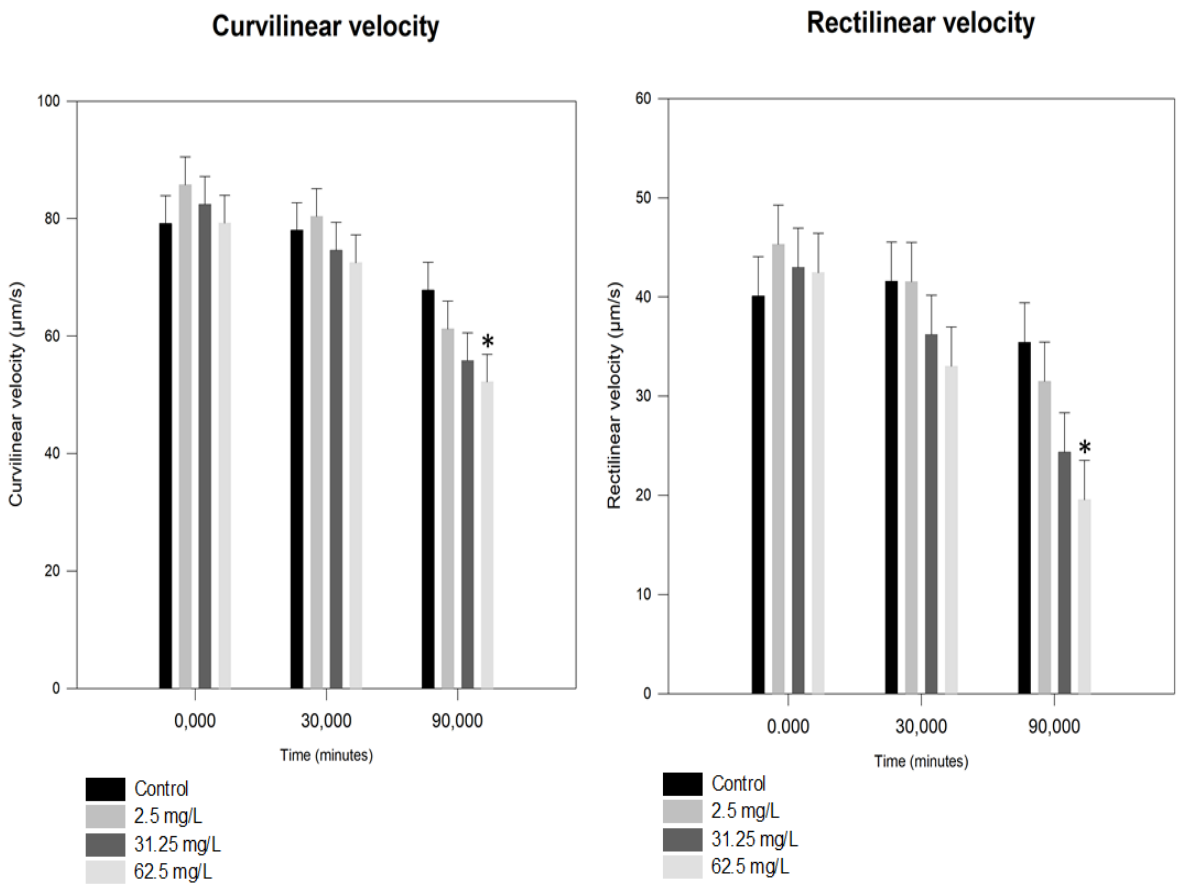

Figure 9: Curvilinear and rectilinear velocity (μm/s) of bovine spermatozoa after being exposed to different Cu concentrations. * represent significant differences between Cu concentrations and the respective control ($p<0.05$).

As expected, exposure time also affected the motility, viability and velocity of spermatozoa ($p<0.05$; Figures 8, 9 and 10). Overall, the largest differences were observed for the highest tested copper concentration (control: 7-22% and 62.5mg/L Cu: 9-42%, for 30 and 90 minutes, respectively).

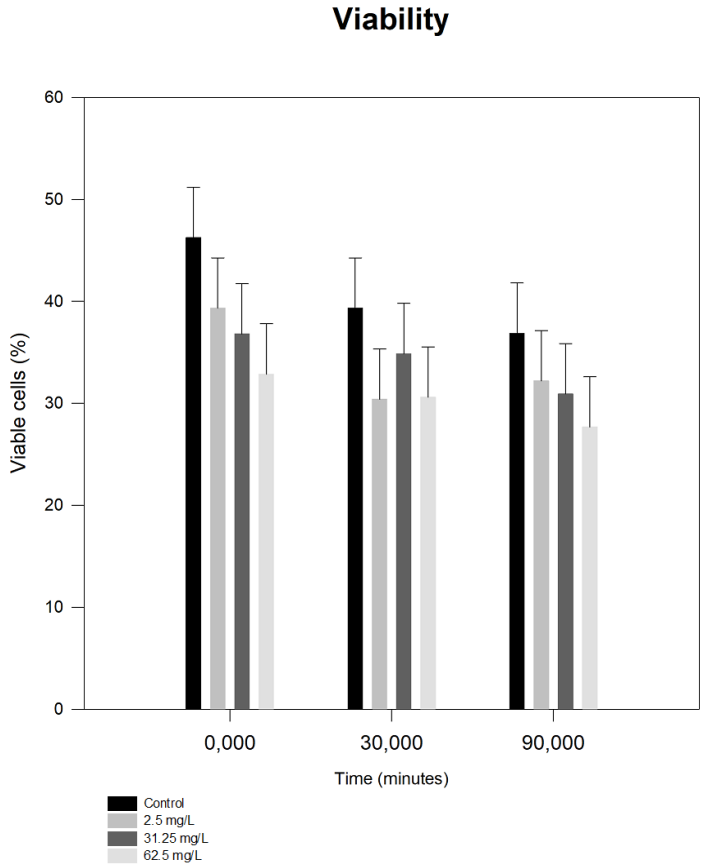

Figure 10: Percentage of viable sperm cells after being exposed to different Cu concentrations.

3.2 *In vitro* assays with glyphosate

Significant effects of glyphosate on the total and progressive motility of spermatozoa started to be observed after 30 minutes of exposure at the highest tested concentration ($p<0.001$; Figure 11). Increasing exposure time, caused a higher effect in spermatozoa motility as significant effects were observed at the two highest tested concentrations (percentage of effect was higher than 50%) ($p<0.001$; Figure 11).

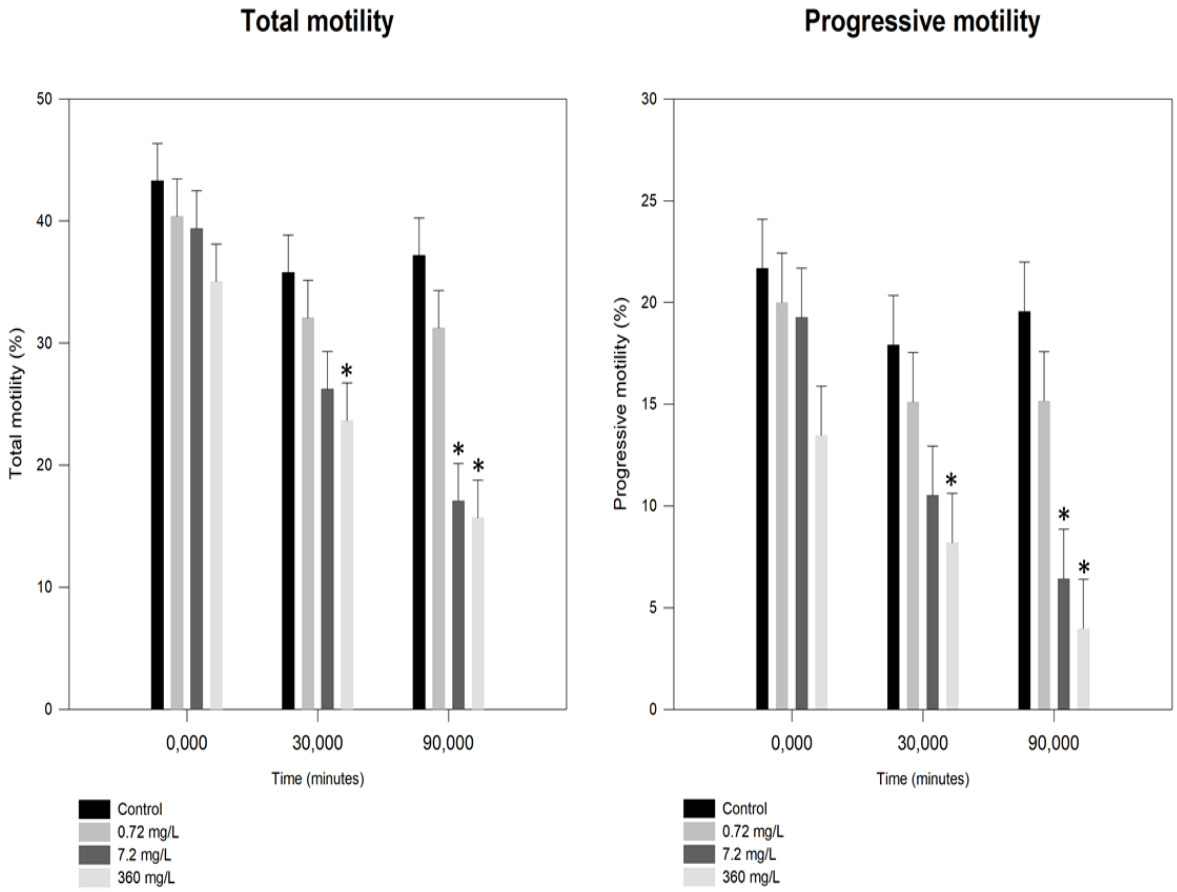

Figure 11: Percentage of total and progressive motility of bovine spermatozoa after being exposed to different glyphosate concentrations. * represent significant differences between glyphosate concentrations and the respective control ($p<0.05$).

Only the highest concentration of glyphosate that was tested significantly reduced the rectilinear and curvilinear velocity of spermatozoa (percentage of effect was higher than 30%) ($p<0.001$; Figure 12).

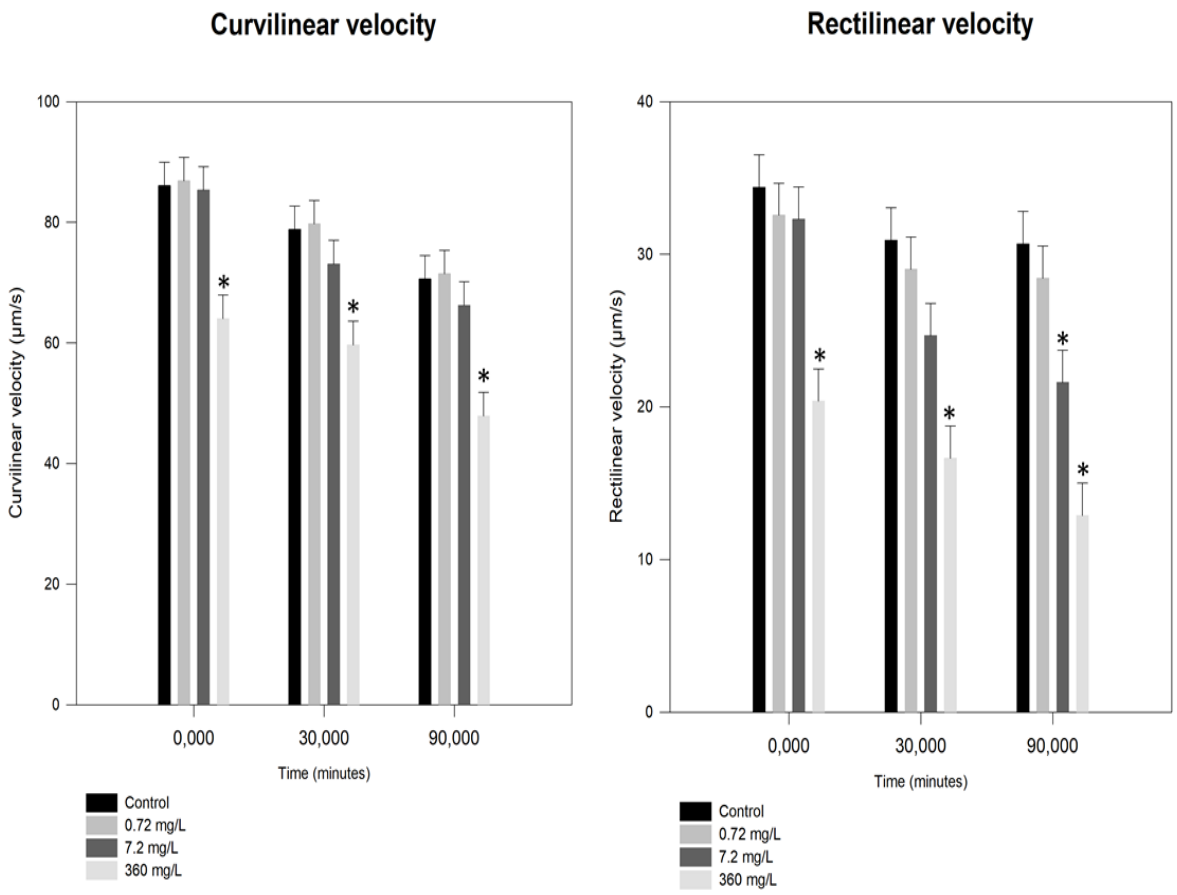

Figure 12: Curvilinear and rectilinear velocity (μm/s) of bovine spermatozoa after being exposed to different glyphosate concentrations. * represent significant differences between glyphosate concentrations and the respective control ($p<0.05$).

The viability of spermatozoa was immediately affected by the highest concentration of glyphosate ($p<0.001$; Figure 13). Also, after a 90 minutes exposure period the three tested concentrations induced a significant reduction in the percentage of viable spermatozoa ($p<0.001$; Figure 13).

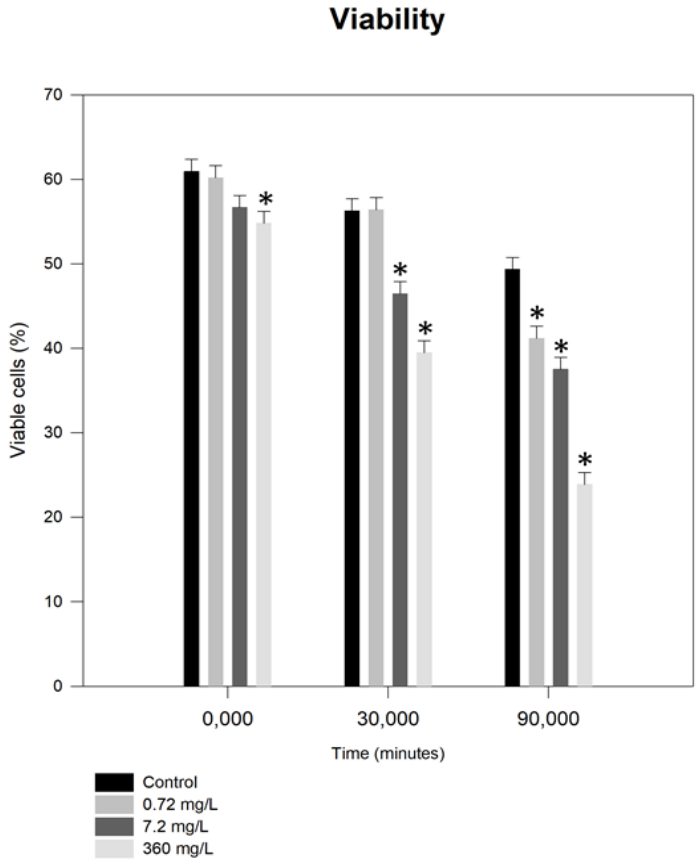

Figure 13: Percentage of viable sperm cells after being exposed to different glyphosate concentrations. * represent significant differences between glyphosate concentrations and the respective control ($p<0.05$).

Exposure time influenced the measured parameters ($p<0.05$; Figures 11, 12 and 13), which decreased with increasing exposure period. The bigger differences were also registered for the highest glyphosate concentration (control: 17-14% and 360mg/L of glyphosate: 35-34%, for 30 and 90 minutes, respectively).

Discussion and Conclusion

4.1 Discussion

The objective of this dissertation was to evaluate the effects of two widely used pesticides, copper sulfate and glyphosate (as the commercial formulation Roundup®), on the motility, velocity and viability of bovine sperm cells. This *in vitro* methodology was used as a surrogate of animal experimentation to preliminarily assess the possible effects of these two pesticides in the reproduction of bovine species. The obtained results showed a decrease for all analyzed parameters in the controls and pesticides concentration over exposure time. These were expected results since spermatozoa exhibit a high sensitivity to *ex vivo* conditions namely to the loss of exogenous energy sources (Mostafapor and Ardebili, 2014, Tsujii et al., 2006). Also, the decreases registered in the present study are within the values already reported by other studies (e.g. Kňažická et al. 2012b, Lukac et al. 2013, 2011, Tabassomi and Alavi-Shoushtari 2013, Tvrdá et al. 2015). For example, Kňažická et al. (2012b) evaluated the effects of copper on bovine spermatozoa over time. These authors registered a decrease in the motility of spermatozoa ranging from 10-16 to 25-70% after an exposure period of 1 and 24h, respectively, under different control media. Also, Lukac et al. (2013) , when studying the effects of nonyphenol on bovine spermatozoa also reported a decrease in motility of spermatozoa ranging from 10 to 16% after an exposure period of 4 or 6 hours, respectively. Though such decreases in motility, velocity, viability of spermatozoa over time under control conditions, this *in vitro* methodology, coupled with the use of Computer-assisted semen analysis (CASA), for the assessment of bovine spermatozoa have already been used and proved to be adequate to evaluate the reproductive toxicity of chemicals (Lukac et al., 2013, Tegelenbosch-Schouten et al., 2006). It allows a reliable assessment of sperm motion parameters, and tests are easily repeatable in other laboratories (Contri et al., 2010).

Motility is an important characteristic for sperm cells since a successful fertilization depends on it, among other parameters. The sperm must be able to swim forward and simultaneously fast enough to pass through the cervical mucus, in order to reach the

oocyte and fertilize it (Nagy et al., 2015). Chemicals, namely pesticides, may impair these parameters and, thus, influence the capacity of the spermatozoa to fertilize the oocyte. Various studies report that the excess intake of copper (concentrations ranging from 0.006 to 7.4 mg/L) can have negative effects on several different species (Araújo et al., 2014, García-Muñoz et al., 2009, Santos et al., 2013, Shuhaimi-Othman et al., 2013, Wang et al., 2015). It can affect the reproductive system of humans and mammals causing degenerative changes in organs and interfering with important reproductive cell functions (copper concentrations ranging from 10 ng/ml to 100μg/ml, i.e. 0.01-100 mg/L) (Roblero et al., 1996, Roychoudhury et al., 2010, 2015). Regarding copper sulfate, the main component that is responsible for its toxicity are the copper ions (U.S. Environmental Protection Agency, 2009b). In the present study, copper concentrations as high as 62.5mg/L did not significantly affected the motility and viability of bovine spermatozoa. But, significant effects were observed at this concentration regarding the velocity. Studies from the scientific literature reported concentrations of copper lower to the ones tested here to induce significant effects on spermatozoa. Kňažická et al. (2012b) reported a significant reduction in motility of bovine spermatozoa after exposure for 1 h to 62.5 or 125μM of copper (corresponding to 3.9 and 7.9 mg/L of copper, respectively), depending on the dilution media, while for spermatozoa viability copper concentrations as low as 3.9μM (corresponding to 0.3mg/L) induced a significant decrease in this parameter. In another study, Kňažická et al. (2013) observed negative correlations between the percentage of motile spermatozoa and progressive motility with the concentrations of copper (ranging from 2.14-6.89 μM /mL, i.e. 0.1-0.4mg/L) in the bovine seminal plasma. Also, Tabassomi and Alavi-Shoushtari (2013) showed that a copper concentration of 0.016mg/L caused deleterious effects on the sperm of the domestic Asian water buffalo *Bubalus bubalis*. When comparing our data with the sensitivity of spermatozoa from other vertebrate species including humans, the results here obtained show a intermediate tolerance to copper of the tested bull spermatozoa. For example, Roychoudhury et al. (2010) reported toxicity of copper sulfate to rabbit sperm cells at doses as low as 3.7 μg/ml (corresponding to 1.48mg/L of copper), while Roblero et al. (1996) studied copper toxicity on human spermatozoa *in vitro* and didn't report significant

effects at copper concentrations of 100μg/ml (100mg/L of copper).

Furthermore, the results obtained in the present study revealed a slight increase (though not statistically significant) of motility and velocity spermatozoa immediately (time zero) after being exposed to the lowest tested concentration of copper (2.5mg/L). This pattern of response was also observed by other authors, though at lower concentrations of copper. Kňažická et al. (2012b) reported that, at a concentration of 7.80 μM/L of $CuSO_4{\cdot}5\,H_2O$ (corresponding to 0.5mg/L of copper), bovine spermatozoa had exhibited a motility and velocity higher than the control. As well, Tabassomi and Alavi-Shoushtari (2013) reported that low copper sulfate concentrations might be used to supplement semen extenders before cryopreservation. These authors reported that adding 0.032 mg/L $CuSO_4 \cdot 5\,H_2O$ (corresponding to 0.008mg/L of copper) to semen extenders improved antioxidant capacity and increases motility and viability after freeze thawing.

Overall the toxicity data of copper on bull spermatozoa suggest a low impact of copper exposure on bull reproduction since environmental relevant concentrations of copper are in general lower than the ones that exerted significant effects in bull spermatozoa. Environmental levels of copper in surface water are usually found at μg/L, also levels of copper in semen or milk of bovines that has been reported in the literature do not reach values within the range of mg/L (e.g. Dickson and Hunter 1981, Kňažická et al. 2012b, Máchal et al. 2002, Muita et al. 2012, Van Genderen et al. 2008).

Regarding glyphosate, all tested concentrations significantly influence at least one of the parameters monitored in the spermatozoa. The viability of spermatozoa was significantly impaired at glyphosate concentrations as low as 0.72mg/L. In the scientific literature there are no *in vitro* toxicity data of glyphosate to bull spermatozoa. However, works performed with spermatozoa of other vertebrate report diverse sensitivities to glyphosate. For example, Yousef et al. (1996) reported that a concentration of 10μM of glyphosate (corresponding to 1.69mg/L) significantly reduced the motility of rabbit spermatozoa after being exposed *in vitro* for 120 minutes. These authors also reported as the median inhibitory concentration for sperm motility index of human and rabbit sperm, after 60 minutes of exposure, 48.2-740 and 23.3-500 μM of glyphosate (corresponding to 8.1-125mg/L and

3.9-84.5mg/L), depending on the medium composition. Clair et al. (2012) reported that concentrations higher than 1000 ppm of glyphosate induced apoptosis of germ cell and Sertoli cells of rats. Anifrandis et al. (2016) observed a significant reduction on human spermatozoa progressive motility 1 hour after being exposed to 0.36mg/L of glyphosate. Anifrandis et al. (2016) also reported mitochondria dysfunction in spermatozoa exposed to 0.36mg/L of glyphosate, suggesting that it was related with the decreased motility since mitochondria produce the energy necessary for cell movement in spermatozoa. When the source of energy is affected, motility is impaired, and velocity is reduced. Furthermore, Cavalli et al. (2013) studied the effects of glyphosate (as Roundup®) on Sertoli cells of rats and concluded that 36mg/L of glyphosate induced oxidative stress in these cells, leading to the activation of stress-response pathways and cell death.

The values of glyphosate that were identified in the present study to significantly induce effects in bull spermatozoa are ecologically relevant. Environmental concentrations of glyphosate that have been measured in surface waters ranging from 0.02 to 5153μg/L (Kjaer et al., 2004, Scribner et al., 2007, Tsui and Chu, 2008, World Health Orgnization, 2005). So it is foresee that bovine may be exposed at glyphosate concentrations that may impair the viability of spermatozoa and consequently reproduction of these vertebrates.

4.2 Conclusion

Overall the toxicity data of copper and glyphosate for bull spermatozoa suggest that only the later may constitute a risk to bull reproduction at environmentally relevant concentrations. However, further studies should be carried out to confirm these results. Namely, further *in vitro* laboratory assays and field studies (establishing a relationship between environmental exposure and effects in the sperm) should be collected to allow a higher relevancy and accuracy in these risk predictions. As well, these *in vitro* toxicity data should be compared with field data in order to further validate as a sensitive tool to assess adverse effects posed by contaminants in the reproduction of bovine. However, not only livestock is affected by these pesticides. Toxicity and reproductive studies should also be carried out for other species present in application sites (like birds, amphibians, and small mammals).

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